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# Preparation and Biological Evaluation of Silybin Liposomes for the Treatment of Liver Disorders

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** The aim of the present study was to develop silybin liposome by incorporating phosphatidyl choline & cholesterol so as to increase its oral bioavailability and liver targeted enhanced hepatoprotection.
**Methodology:** Thin film hydration technique was used for the development of liposomes by using phosphatidyl choline, cholesterol and drug. Liposomes were evaluated for vesicle size, zeta potential, PDI, encapsulation efficiency, surface morphology and *in vitro* drug release study. Further the optimized formulation was evaluated for APAP-induced alterations in liver and kidney function tests in rats and histopathological studies.
**Results:** The results were promising with a sustained drug release of 80% within 20hrs, optimized vesicle size of 276nm and 89% encapsulation efficiency. The animal studies demonstrated superior hepatoprotective effect compared to silybin solution.
**Conclusion:** The silybin liposomes showed better *in-vitro* release & *in-vivo* hepatoprotection along with better animal activity & improvement in histopathological changes as compared to silybin.

*Keywords: Silybin; liposome; hepatoprotection; phosphatidyl choline; lipid.*

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## 1. INTRODUCTION

Silymarin (SM) is an herb obtained from *Silybum marianum* possessing hepatoprotective activity for the treatment of liver disorders. This herb consists of a mixture of Silybin, Isosilybin, Silydianin, and Silychristin. Silybin is one of the major flavonolignans from the mixture & consists of about 60-70% of silymarin. Silybin is a mixture of two diastereomers (A, B) which are present in a ratio of 1:1 [1,2]. Silybin is a known hepatoprotectant but due to its low serum, tissue levels, low solubility & absorption lead to its poor oral bioavailability [3,4]. As per literature, many research studies have been performed, such as solid dispersions, porous silica nanoparticles, lipid complexes, etc so as to improve the solubility & bioavailability of Silybin [5-10], but none of the methods were useful to achieve a sustainable bioavailability.

Liposomes have been used effectively to improve the solubility & bioavailability of poor water soluble drugs. This dosage form does not target the liver & inflammatory cells selectively, and can be used as an alternate formulation to improve bioavailability of silybin. It can be prepared by various methods such as thin-film dispersion, reversed phase evaporation, spray-freeze drying, etc [11-15]. Liposomes consist of lipid bilayers with a water phase as inner layer which encapsulates the water-soluble & lipophilic drugs to improve the efficacy & safety of drugs. Liposome formulations are an excellent dosage forms to improve the shortcomings of existing silybin formulations by increasing its oral bioavailability [16].

## 2. MATERIALS AND METHODS

All the material was of analytical grade & obtained from Merck. Chemicals, excipients & drugs (Silybin, Cholesterol & Phosphatidyl choline) were purchased from Sigma Aldrich, India. Paracetamol/APAP was obtained as a gift samples from Symed Pharmaceutical Pvt. Ltd, Hyderabad, India. 1,1′,3,3′-Tetraethoxypropane, Crystalline beef liver catalase, reduced GSH, and 5,5′-Dithiobis (2-nitrobenzoic acid) were purchased from SD Fine Chemicals, Mumbai, India. Sulphanilamides, Naphthylamine diamine HCl, and Phosphoric acid were obtained from Loba Chemi Pvt. Ltd., Mumbai, India. Rats-specific tumor necrosis factor-α (TNF-α), Interleukin (IL)-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) Kit were obtained from Bethyl Laboratories Inc. (Montgomery, TX, USA).

### 2.1 Pre-formulation Studies

Silybin was evaluated for its physicochemical interaction with Phosphatidyl choline (PC) and Cholesterol (C) at a ratio of 1:1:1 using differential scanning calorimetry (DSC).

#### 2.1.1 Solubility studies

Solubility studies were conducted in different solvents & Phosphate buffer (pH 6.8 & pH 7.4) using shake flask method, wherein, excess of the drug was dissolved in 30 mL of the solvent in a stoppered flask and the flasks were allowed to shake for 24 h. The resulting solution was then filtered and samples were analyzed using UV spectrophotometer.

#### 2.1.2 Drug –excipient compatibility studies

The drug-excipient compatibility studies were conducted using Fourier Transform Infrared (FTIR) spectroscopy & a spectrum of the liposome was compared with the standard drug.

#### 2.1.3 Development of silybin-liposomes

Lipid film hydration technique was used for the preparation of liposomes wherein, Silybin, Phosphatidyl choline & Cholesterol were taken into a round bottom flask & dissolved in methanol-chloroform mixture (1:9) [7]. Excess solvent was evaporated under vacuum at 40°C in a rotary evaporator to obtain a thin film. The film was kept for drying overnight in a vacuum desiccator so as to remove the solvent traces. The film was hydrated with phosphate buffer saline (pH 7.4), at 100 RPM and at 50°C for 1 h to prepare a liposomal suspension. The liposome vesicle size was reduced by high-pressure homogenization at 20,000 psi for 5 cycles and were kept overnight in deep freezer at -80°C. The frozen liposomes were lyophilized at reduced pressure and stored at 4°C in airtight containers for further experiments (Table 2).

### 2.2 Physicochemical Characteristics of Liposomes

#### 2.2.1 Particle size and zeta potential

Mean particle size, polydispersity index (PDI) and zeta potential of liposomes were determined by Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) after suitable re-dispersion in water.

#### 2.2.2 Encapsulation efficiency in liposome

Liposomal suspension of each formulation was centrifuged at 1000 rpm for 10 min to separate entrapped particles. Supernatant was collected and again centrifuged at 64,000 x g at 4–8°C for 60 min (Sigma centrifuge, SciQuip Ltd., UK). Supernatant was separated and stored for testing free drug content. To the pellet, 100 ml of 10% Triton X and 900 ml of methanol were added. The concentration of silybin in supernatant and precipitate was analyzed by reversed phase high performance liquid chromatography (RP-HPLC). The HPLC column was Hibar1 RP C-18, 4.6 mm x 250 mm from Merck, Darmstadt, Germany. The mobile phase methanol: water (50:50, pH 3.5) was pumped at a flow rate of 1.0 ml/min. The detection wavelength and the detection limit of silybin adopted were 286 and 1 µg/ml respectively.

#### 2.2.3 Morphology studies

To examine the morphology, SEM studies were conducted by placing the sample on tape which was fixed on dies. Then vacuum was applied at high for taking pictures under high as well as low voltage.

### 2.3 *In vitro* Drug Release Studies

The *in vitro* release study was performed in a diffusion cell set-up across a dialysis membrane. An inverted cylindrical test tube cut to a height of 8 cm was used as a donor compartment. The receiver compartment consisted of 100 ml of phosphate buffer (pH 7.4, 37 °C) in a beaker placed over a water bath. A dialysis membrane which was pre-soaked in warm water for 30 min was placed at the lower end of the cylindrical setup and the membrane separated the donor compartment from the receiver compartment. Liposomes containing 20 mg of drug was suspended into 5 ml of pH 7.4 buffer and placed in the donor compartment. The system was stirred using a magnetic stirrer and bead. Samples (5 ml) were removed from the receiver compartment and replaced with the same volume of fresh medium immediately. The samples were analyzed at 287 nm.

### 2.4 Animal Studies

Adult Wistar rats (180–220 g) were obtained and housed in cages in a facility maintained at 24 ± 1°C, with a relative humidity of 45–55% and 12:12 h dark/light cycle. The animals had free access to standard pellet chow and filtered water throughout the experimental period. All experiments were carried out between 09:00 and 17:00 h. Animals were transferred to the testing laboratory 1 h before the experiment for adaptation.

#### 2.4.1 Induction of APAP-induced toxicity and drug treatment schedule

Induction of APAP-induced hepatic and renal toxicity was carried out according to a previously reported method [16]. Briefly, APAP was administered to rats orally at a dose of 700 mg/kg [17]. Fasted rats were randomly divided into 5 groups,

G1- Normal: Rats were administered a single daily dose of distilled water (10 mg/kg), p.o. for 14 days. They did not receive any dose of APAP. G2 -APAP control: Rats served as the model control were administered a single daily dose of distilled water (10 mg/kg, p.o.) 2 h before oral administration APAP suspension (700 mg/kg) for 14 days. G3 -Silybin (100): Rats were administered a single daily dose of silybin (100 mg/kg, p.o.) in distilled water, 2 h before oral administration APAP suspension (700 mg/kg) for 14 days. G4 - Silybin Lipo (100): Rats were administered a single daily dose of Silybin liposomes (equivalent to 100 mg/kg, p.o.) 2 h before oral administration APAP suspension (700 mg/kg) for 14 days. G5-Placebo Lipo (100): Rats were administered a single daily dose of placebo liposomes (100 mg/kg, p.o.) for 14 days, they did not receive any dose of APAP.

After 14 days of treatment, rats fasted overnight were sequentially anesthetized with anesthetic ether for about 30-40 s. The blood was withdrawn by the retro-orbital puncture. Each blood sample was collected into separate vials for the determination of serum parameters. After blood collection, the animals were sacrificed by cervical dislocation, and then liver as well as kidney were isolated. The specimens were divided into two portions: one portion was used for biochemical estimation, and another portion was processed for histopathological examination.

#### 2.4.2 Determination of hepatic pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) levels

Levels of hepatic TNF-α, IL-1β, and IL-6 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction (Bethyl Laboratories Inc., Montgomery, TX,

USA). TNF-α, IL-1β, and IL-6 were determined from a standard curve for the combination of these cytokines. The concentrations were expressed as pg/mg of protein. Briefly, the quantifications of TNF-α, IL-1β, and IL-6 were done with the help and instructions provided by Bethyl Laboratories Inc Rat TNF-α, IL-1β, and IL-6 immunoassay kit. The Rat TNF-α, IL-1β, and IL-6 immunoassay was a 4.5 h solid-phase designed to measure rat TNF-α, IL-1β, and IL-6 levels. The assay employed a sandwich enzyme immunoassay principle. A monoclonal antibody specific for rat TNF-α, IL-1β, and IL-6 was pre-coated on the microplates. Standard, control, and samples were added into the wells by a pipette, and any rat TNF-α, IL-1β, and IL-6 present in the sample was thus bound by the immobilized antibody. After washing away the unbound substance, an enzyme-linked polyclonal antibody specific for rat TNF-α, IL-1β, and IL-6 was added via a pipette into the microtitre wells. Any unbound antibody was washed off, and then a substrate solution was added to the wells. The enzymatic reaction produced a blue product that turned yellow when the stop solution was added. The intensity of the color generated was measured, which was proportional to the amount of rat TNF-α, IL-1β, and IL-6 bound in the initial steps. A standard curve was run on each assay plate using recombinants of the TNF-α, IL-1β, and IL-6 in serial dilutions. The sample values were then read, and calculations were made according to the standard curve. Values were expressed as means ± SEM. The levels of TNF-α, IL-1β, and IL-6 were expressed as units per mg of protein.

#### 2.4.3 Histopathological examination

Liver and kidney tissues were stored in 10% formalin for 24 h. The specimen was dehydrated and placed in Xylene for 1 h (three times) and later in Ethyl alcohol (70, 90, and 100%) for 2 h. The infiltration and impregnation were carried out by treating with Paraffin wax twice, each time for 1 h. Tissue specimens were cut into sections of 3-5 mm thick and stained with Hematoxylin and Eosin (H&E). The specimen was mounted on the slide using Distrene Phthalate Xylene (DPX) as a mounting medium. Sections were examined under a light microscope for the inspection of the histopathology features of the specimen and infiltration of cells. The various changes in histological features were graded as Grade 0 (not present or very slight), Grade 1 (mild), Grade 2 (moderate), and Grade 3 (severe) as described earlier [18].

#### 2.4.4 Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Data analysis was performed using software (v 5.0, Graph Pad, San Diego, CA). Data of biochemical parameters were analyzed using one-way analysis of variance (ANOVA) and followed by Tukey's multiple range tests for each parameter separately. Data of the histological score were analyzed using nonparametric one-way ANOVA followed by the Kruskal-Wallis test for post hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Pre-formulation Studies

Physical mixtures (Silybin and lipids) did not show any drug- lipid interactions and were found to be stable, physically and chemically.

#### 3.1.1 Solubility studies

The solubility of Silybin in different solvents like Water, Ethanol, pH 6.8 phosphate buffer and pH 7.4 phosphate buffer were mentioned in table 1. From the study it was found that Silybin was practically insoluble in water, sparingly soluble in ethanol and soluble in pH 6.8 and pH 7.4 phosphate buffers. The formulation had increased the solubility of Silybin as compared to Silybin alone solubility.

**Table 1. Solubility of silybin in different solvents**

| Solvent | Solubility |
|---|---|
| Water | 50 µg/ml |
| Ethanol | 0.1 mg/ml |
| pH 6.8 phosphate buffer | 0.5 mg/ml |
| pH 7.4 phosphate buffer | 0.6 mg/ml |

#### 3.1.2 Preparation of silybin liposomes

The encapsulation percentage of silybin was found to be maximum for formulation containing Phosphatidyl choline and Cholesterol at molar ratio 9:1 (Table 1). Particle size obtained from sonication and high-pressure homogenizer (HPH) was compared. More homogeneous and uniform particles were obtained by HPH compared to sonication alone. A pressure of 20,000 psi for 5 cycles was found to be optimum for size reduction. This was selected as optimized formulation.

### 3.1.3 Drug –Excipient compatibility studies

The formation of silybin liposome was confirmed by FTIR spectroscopy comparing the spectrum of liposome complex with the individual component. There was a significant difference between the complex & individual component. The spectrum of silybin complex showed a new peak at 1455 $cm^{-1}$, which did not appear in the individual components spectra of silybin & phosphatidylcholine. The spectrum also showed a decreased intensity in the peak at 1733 $cm^{-1}$. The disappearance of broad -OH peak from silybin complex further confirmed the formation of complex. These results suggested the formation of hydrogen-bonding between silybin & phosphatidylcholine, thus causing significant difference in the intensity of functional group involved in the interaction (Fig. 1 and 2).

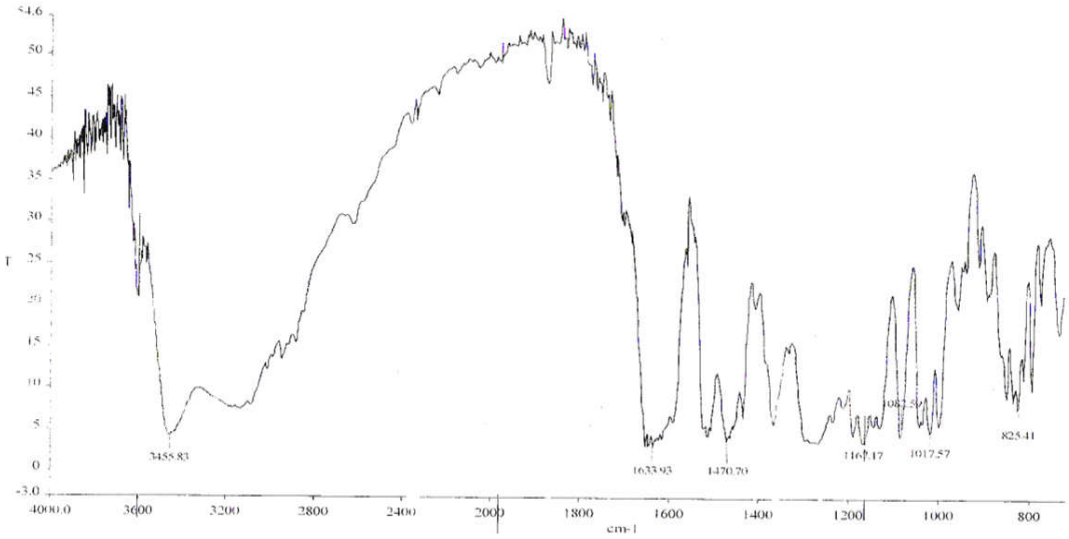


**Fig. 1. FTIR of silybin**

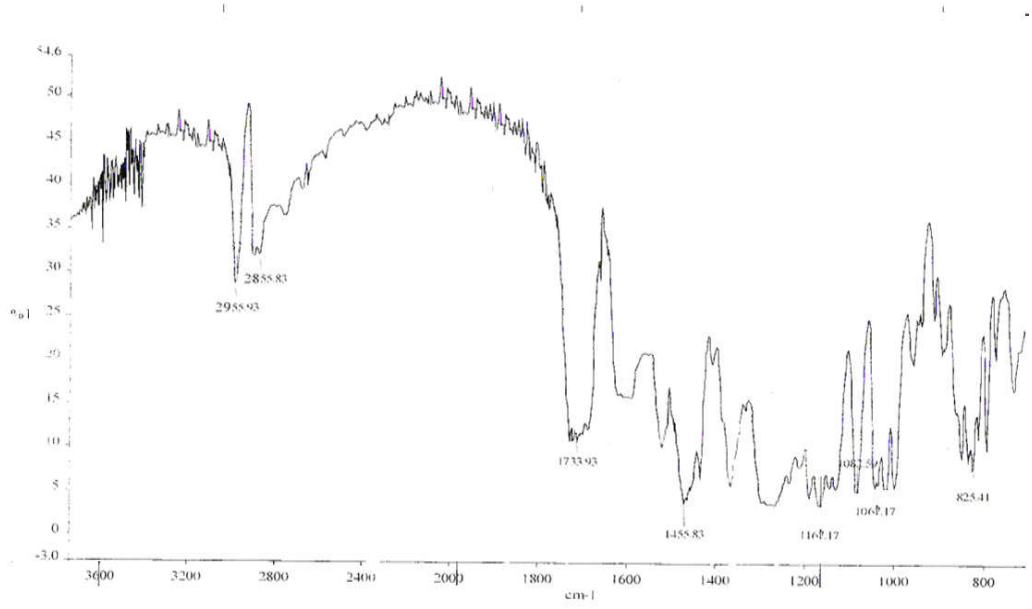


**Fig. 2. FTIR of silybin liposome**

## 3.2 Physicochemical Characteristics of Liposomes

### 3.2.1 Vesicle size and entrapment efficiency

Vesicle size and Entrapment efficiency was estimated for different ratios of Liposomes with different drug concentration. LP6 and LP9 having Lipid: Cholesterol ratio 9:1 and 9:2 with 60mg drug concentration showed highest % Entrapment efficiency. Vesicle size of both the ratios was observed as 276±3.45 and 402±3.67 respectively (Table 2). The higher entrapment efficiency was observed for higher drug concentration (60 mg) along with suitable Lipid: Cholesterol ratio, thus it can be concluded that high drug concentration leads to greater entrapment efficiency. The optimized Silybin liposome was spherical in shape and homogeneous in particle size distribution. Zeta potential of formulation (-35±1.89) was found to be more than optimum for physical stability of the formulation.

### 3.2.2 Zeta potential and PDI of liposomes

Zeta potential of all 9 formulation was between -31±1.25 to -36±1.34. LP4 showed highest zeta potential value of -36±1.34 and LP1 showed lowest zeta potential value of-31±1.25. PDI was in range of 0.13 to 0.25. LP8 showed highest PDI value whereas LP1 showed lowest PDI value (Table 3).

### 3.2.3 Scanning electron microscopy

After slightly shaking in water, a monolayer formed and the particles appeared to be spherical in shape (Fig. 3). The DSC thermogram of silybin showed an endothermic peak at 252.83°C while for liposomes it was at 211.62°C, thus suggesting a possible interaction with lipid components & accounting for enhanced entrapment.

### 3.2.3 In-vitro drug release studies

The release of liposomal Silybin formulation exhibited biphasic release pattern with an initial burst release followed by a sustained pattern. The initial burst release was because of the unentrapped drug. From the graph it was found that, the drug solution exhibited only 30% of drug release within the time period of 20hrs, where as the Silybin liposomal formulation released 80% of the drug with a sustained pattern (Fig. 3). Thus the encapsulation of Silybin in liposomes increased the solubility and sustained the drug release.

**Table 2. Vesicle size and entrapment efficiency**

| Code | Lipid :cholesterol | Drug | Vesicle size (nm) | %Entrapment efficiency |
|---|---|---|---|---|
| LP1 | 9:0 | 20mg | 135±2.56 | 31.5±1.23 |
| LP2 | 9:0 | 40mg | 300±3.64 | 68.3±1.56 |
| LP3 | 9:0 | 60mg | 560±2.78 | 79.5±1.32 |
| LP4 | 9:1 | 20mg | 137±1.54 | 59.6±1.67 |
| LP5 | 9:1 | 40mg | 321±2.53 | 75.3±1.89 |
| **LP6** | **9:1** | **60mg** | **276±3.45** | **89.2±2.45** |
| LP7 | 9:2 | 20mg | 142±2.96 | 57.3±2.56 |
| LP8 | 9:2 | 40mg | 349±1.99 | 73.4±2.67 |
| **LP9** | **9:2** | **60mg** | **402±3.67** | **81.5±1.89** |

**Table 3. Zeta potential and PDI of liposomes**

| Formulation | PDI | Zeta potential |
|---|---|---|
| LP1 | 0.13 | -31±1.25 |
| LP2 | 0.21 | -33±1.42 |
| LP3 | 0.18 | -34±1.56 |
| LP4 | 0.24 | -36±1.34 |
| LP5 | 0.17 | -32±1.78 |
| LP6 | 0.21 | -35±1.89 |
| LP7 | 0.19 | -33±1.32 |
| LP8 | 0.25 | -32±2.15 |
| LP9 | 0.21 | -34±1.56 |

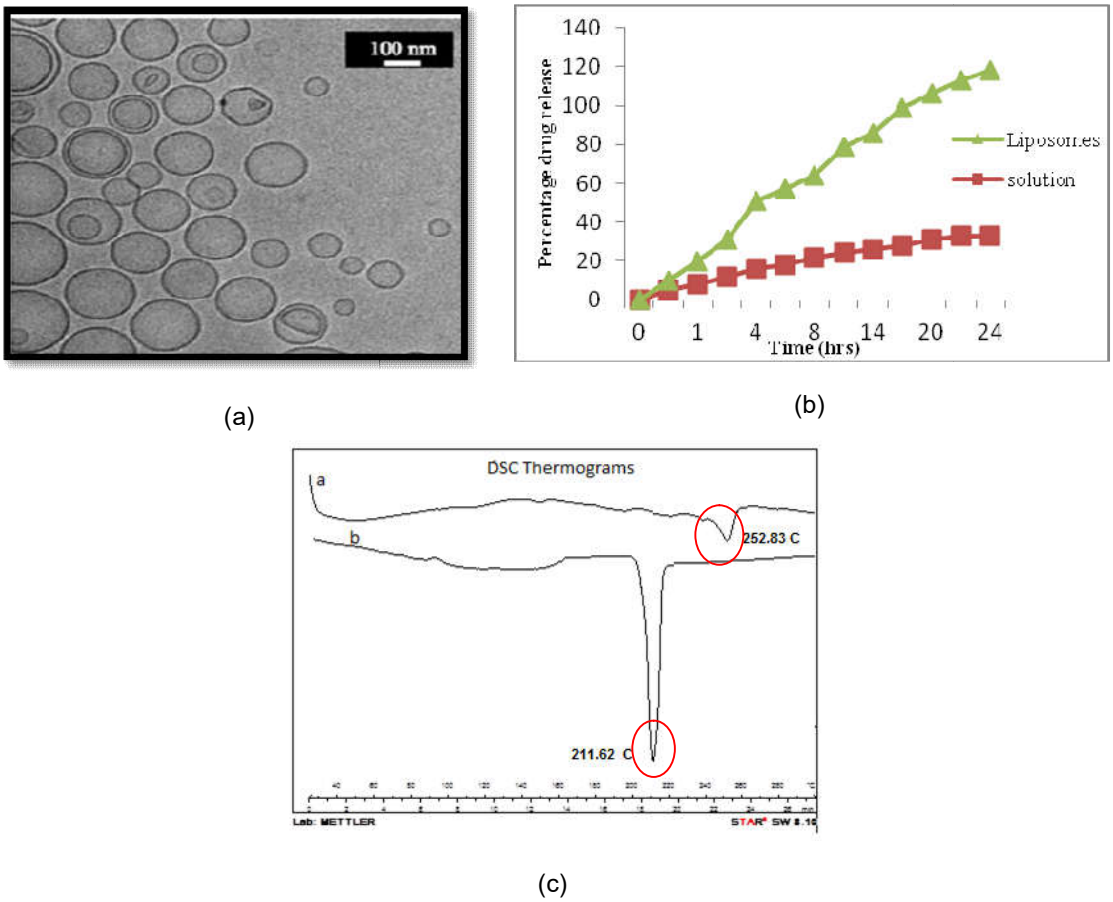


**Fig. 3. Characterization & in-vitro release study of Liposome; (a) SEM image, (b) release study graph of liposome, (c) DSC thermogram**

**Table 4. Stability study of liposomes**

| Condition | Time interval | Appearance | Size (nm) | Entrapment efficiency(%) |
|---|---|---|---|---|
| Refrigeration (5±3°C) | 1month | Milky suspension | 267±2.45 | 89.2±2.45 |
| | 2month | Milky suspension | 263±3.45 | 88.2±3.45 |
| | 3month | Milky suspension | 264±2.45 | 86.3±2.55 |
| Room Temperature ($25\pm2^0$C) | 1month | Milky suspension | 273±3.45 | 88.2±2.35 |
| | 2month | Slight coagulation | 291±1.56 | 80.5±2.48 |
| | 3month | coagulation | 312±3.21 | 71.2±2.47 |
| Accelerated (40±2°C/75 ±5% RH) | 1month | Milky suspension | 276±3.45 | 81.6±2.47 |
| | 2month | coagulation | 298±3.56 | 71.3±3.67 |
| | 3month | Coagulation with lumps | 396±1.65 | 63.5±1.32 |

## 3.3 Stability Study of Liposomes

Liposome stability study was conducted in three different conditions, Refrigeration (5±3°C), Room Temperature ($25\pm2^0$C) and Accelerated (40±2°C/75 ±5% RH)for 1, 2 and 3 month. In Refrigerated condition appearance of Liposome was like Milky suspension with size ranging from 263±3.45 nm to 267±2.45 nm and Entrapment efficiency % as 86.3±2.55 % to 89.2±2.45%. At Room temperature appearance was milky suspension in 1 month whereas slight coagulation in 2 and coagulation in 3 months respectively, with its size ranging from

273±3.45nm to 312±3.21nm and Entrapment efficiency % as 71.2±2.47% to88.2±2.35%. Under accelerated conditions, the suspension was milky in 1 month and coagulation in 2 and coagulation with lumps in 3 months respectively, with its size ranging from 276±3.45nm to 396±1.65nm and Entrapment efficiency % as 63.5±1.32% to 81.6±2.47% (Table 4).

Bioavailability studies of the liposome in male Wistar rats exhibited good results as compared to Silybin. $AUC_{0-2}$ of formulation was more than three and half times higher than silybin suspension. The $C_{max}$ obtained from the bioavailability study was 140.25±20.45 ng/ml for silybin, with a $t_{max}$ of 0.5 h. The $C_{max}$ was increased by liposomal formulation to 720.20±40.58 ng/ml without a change in $t_{max}$.

The bioavailability of silybin was increased, wherein $C_{max}$ of liposomal silybin increased more than five times as compared to silybin with no change in $t_{max}$.

## 3.4 Animal Activity

### 3.4.1 Effect of silybin formulation treatment against APAP-induced alterations in body weight, relative liver weight, relative spleen weight, and relative kidney weight in rats

There was significant ($p < 0.001$) decrease in body weight of APAP control rats as compared to normal rats. Whereas relative organ weights of liver, spleen and kidney were significantly ($p < 0.001$) increased in APAP control rats as compared with normal rats. Treatment with silybin (100 mg/kg & silybin liposomes (eq. to 100 mg/kg) showed a significant increase ($p < 0.05$, $p < 0.001$, $p < 0.01$ and $p < 0.001$, respectively) in body weight as compared to APAP control rats (Table 4). Administration of silybin (100 mg/kg), silybin liposomes (eq. to 100 mg/kg) were effectively ($p < 0.05$, $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) inhibited APAP-induced decreased relative liver weight as compared to APAP control rats. When compared with APAP control rats, relative spleen and kidney weight was effectvively decreased ($p < 0.001$) after treatment with silybin (100 mg/kg), silybin liposomes (eq. to 100 mg/kg). However, relative spleen weight was more effectively ($p < 0.05$ and $p < 0.01$) decreased in silybin liposomes (eq. to 100 mg/kg) treatment as compared to silybin (100 mg/kg). Body weight, relative liver weight, relative spleen weight, and relative kidney weight did not differ in placebo liposomes, and treated groups as compared to the normal group (Table 6).

### 3.4.2 Effect of silybin formulation treatment against APAP-induced alterations in liver function tests in rats

Chronic administration of APAP caused a noticeable ($p < 0.001$) increase in ALP, AST, ALT, total bilirubin, and direct bilirubin in APAP control rats compared to normal rats. When compared with APAP control rats, there was a significant ($p < 0.001$) decrease in the level of alkaline phosphatase after silybin (100 mg/kg), silybin liposomes (eq. to 100 mg/kg). ALP level was more significantly decreased ($p < 0.01$) in silybin liposomes (eq. to 100 mg/kg) treated group as compared to silybin (100 mg/kg) treatment (Table 5). ALT and AST levels were effectively decreased ($p < 0.01$, $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) in silybin (100 mg/kg), silybin liposomes (eq. to 100 mg/kg) as compared to APAP control rats. Silybin liposomes (eq. to 100 mg/kg) treatment more significantly ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) reduced ALT levels as compared to silybin (100 mg/kg) treatment. Silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) treatment effectively ($p < 0.01$, $p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) decreased the total and direct bilirubin. Administration of silybin liposomes (eq. to 100 mg/kg)) more noticeably ($p < 0.01$) reduced elevated levels of total bilirubin level when compared with treatment (100 mg/kg). Liver function tests did not differ in placebo liposomes treated groups compared to the normal group (Table 7).

### 3.4.3 Effect of silybin formulation treatment against APAP-induced alterations in kidney function tests in rats

The level of serum albumin and serum uric acid were significantly ($p < 0.001$) decreased in APAP control rats as compared to normal rats, whereas blood urea nitrogen and serum creatinine level were significantly ($p < 0.001$) increased in the APAP control rats as compared to normal rats. (Table 6).

**Table 5. Pharmacokinetic parameters of Silybin**

| Pharmacokinetic parameters (units) | Silybin Mean±SEM | Liposome Mean±SEM |
|---|---|---|
| $C_{max}$ (ng/ml) | 140.25±20.45 | 720.20±40.58 |
| $t_{max}$ (h) | 0.50±0.0 | 0.50±0.0 |
| $AUC_{0-2}$ (h*ng/ml) | 120.10±6.99 | 440.53±22.95 |
| $AUC_{0-in}f$ (h*ng/ml) | 162.38±6.52 | 503.66±24.20 |
| Half-life (h) | 1.30±0.20 | 0.68±0.04 |
| Clearance (ml/h) | 1276.04±40.52 | 410.76±20.21 |
| Volume of distribution ($V_d$) (ml) | 1972.84±166.43 | 445.32±26.4 |

**Table 6. Effect of silybin formulation treatment against APAP-induced alterations in body weight, relative liver weight, relative spleen weight, and relative kidney weight in rats**

| Treatment | Body weight (gm) | Liver weight (gm) | Liver weight / Body weight ratio | Spleen weight (gm) | Spleen weight / Body weight ratio | Kidney weight (gm) | Kidney weight / Body weight ratio |
|---|---|---|---|---|---|---|---|
| Normal | 248.00 ± 1.65 | 5.46 ± 0.34 | 0.022 ± 0.001 | 0.34 ± 0.03 | 0.0013 ± 0.0001 | 1.16 ± 0.04 | 0.0047 ± 0.0002 |
| APAP Control | 223.20 ± 3.36$^{###}$ | 8.27 ± 0.35$^{###}$ | 0.037 ± 0.002$^{###}$ | 1.01 ± 0.04$^{###}$ | 0.0046 ± 0.0002$^{###}$ | 1.51 ± 0.03$^{###}$ | 0.0068 ± 0.0002$^{###}$ |
| Silybin (100) | 238.80 ± 2.55* | 6.50 ± 0.28** | 0.027 ± 0.001** | 0.78 ± 0.05** | 0.0033 ± 0.0002*** | 1.33 ± 0.03** | 0.0056 ± 0.0002*** |
| Silybin Lipo (100) | 244.00 ± 4.19** | 6.09 ± 0.46*** | 0.025 ± 0.002*** | 0.57 ± 0.03***,$$,& | 0.0023 ± 0.0001***,$ | 1.27 ± 0.03*** | 0.0052 ± 0.0001*** |
| Placebo Lipo | 253.00 ± 5.01 | 4.99 ± 0.22 | 0.020 ± 0.001 | 0.40 ± 0.04 | 0.0016 ± 0.0001 | 1.13 ± 0.03 | 0.0045 ± 0.0001 |

*Data are expressed as mean ± SEM (n = 6) and analysed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. *p <0.05, **p <0.01 and ***p <0.001 as compared to APAP group. $^{###}$p <0.001 as compared to normal group. $^{$}$p <0.05 and $^{$$}$p <0.01 as compared to silybin (100 mg/kg) treated. APAP: Acetaminophen; Silybin Lipo (100): Silybin liposomes (equivalent to 100 mg/kg)*

**Table 7. Effect of silybin formulation treatment against APAP-induced alterations in liver function tests in rats**

| Treatment | Alkaline Phosphatase (IU/l) | Aspartate Transaminase (IU/L) | Alanine Transaminase (IU/L) | Total Bilirubin (mg %) | Direct Bilirubin (mg %) |
|---|---|---|---|---|---|
| Normal | 36.46 ± 0.96 | 133.40 ± 9.01 | 33.11 ± 4.43 | 0.10 ± 0.01 | 0.28 ± 0.02 |
| APAP Control | 228.60 ± 3.79$^{###}$ | 302.80 ± 5.25$^{###}$ | 158.50 ± 4.98$^{###}$ | 0.32 ± 0.01$^{###}$ | 0.65 ± 0.03$^{###}$ |
| Silybin (100) | 111.40 ± 4.88*** | 201.30 ± 10.30** | 92.56 ± 3.71** | 0.23 ± 0.01** | 0.48 ± 0.02** |
| Silybin Lipo (100) | 85.28 ± 3.52***,$$ | 172.30 ± 12.99*** | 55.54 ± 3.78***,$$ | 0.16 ± 0.01***,$$ | 0.40 ± 0.03*** |
| Placebo Lipo | 46.85 ± 3.54 | 132.70 ± 11.06 | 30.73 ± 3.37 | 0.10 ± 0.01 | 0.28 ± 0.02 |

Chronic administration of silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) significantly increased serum albumin and serum uric acid ($p < 0.001$) level as compared to APAP control rats. When compared with silybin (100 mg/kg), silybin liposomes (eq. to 100 mg/kg) more noticeably ($p < 0.01$) increased serum uric acid level.The level of blood urea nitrogen and serum creatinine were significantly decreased by silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) as compared to APAP control rats. Serum creatinine were more efficiently ($p$ <0.01) decrease in silybin liposomes (eq. to 100 mg/kg) treatment as compared to silybin (100 mg/kg) treatment. Placebo liposomes treated groups did not show any significant difference in the kidney function tests compared to the normal group (Table 8).

#### 3.4.4 Effect of silybin formulation treatment against APAP-induced alterations in hepatic pro-inflammatory cytokine levels in rats

There was a significant increase ($p < 0.001$) in hepatic TNF-α, IL-1β, and IL-6 in APAP control rats compared to normal rats. Treatment with silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) showed significant amelioration ($p < 0.001$) in APAP-induced increased in hepatic IL-1β and IL-6 levels as compared to APAP control rats (Table 8). When compared with APAP control rats, silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) treated rats showed a significant decrease in hepatic TNF-α levels. Hepatic TNF-α and IL-1β levels was more noticeably decreased ($p < 0.01$) in silybin liposomes (eq. to 100 mg/kg) as compared to silybin (100 mg/kg) treatment (Table 9). However, placebo liposomes treated groups did not show any significant alterations in hepatic TNF-α, IL-1β, and IL-6 compared to normal rats.

#### 3.4.5 Effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat liver

In the histopathological studies, normal & placebo liposomes treated groups showed normal central vein in liver parenchymal cells without any signs of necrosis (Fig. 4 and Fig.5). However, there was evidence of mild congestion and inflammatory infiltration. The histopathological examination of the liver from APAP administered rats was showed marked ($p$ <0.001) infiltration of inflammatory cells, macrovesicular fatty changes, necrosis, edema, and shrunken hepatocytes with chromatin condensation. However, silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) treatment effectively reduced ($p$ <0.05) APAP-induced alterations in hepatic tissue reflected by moderate histopathological changes in the liver such as mild sinusoidal congestion, cytoplasmic vacuolation and inflammatory cells along with few fatty globules.

Photomicrograph of sections of liver of normal (A), APAP control (B), Silybin (100 mg/kg) (C), Silybin liposomes (100 mg/kg) (D), Placebo liposomes (E).

#### 3.4.6 Effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat kidney

Fig. 6 and Fig. 7, showed the normal architecture of the kidney from normal as well as placebo liposomes treated groups. Chronic administration of APAP caused significant ($p < 0.001$) renal damage evident by glomerular structural disruption, a partial endothelial rupture in the

**Table 8. Effect of silybin formulation treatment against APAP-induced alterations in kidney function tests in rats**

| Treatment | Albumin (mg/dL) | Blood Urea Nitrogen (mg/dL) | Serum Creatinine (mg/dL) | Uric acid (mg/dL) |
|---|---|---|---|---|
| Normal | 3.76 ± 0.18 | 4.45 ± 0.49 | 1.12 ± 0.18 | 3.69 ± 0.25 |
| APAP Control | 1.10 ± 0.25$^{###}$ | 20.39 ± 0.40$^{###}$ | 5.07 ± 0.16$^{###}$ | 1.20 ± 0.20$^{###}$ |
| Silybin (100) | 2.29 ± 0.22* | 13.72 ± 0.54** | 3.01 ± 0.10** | 2.75 ± 0.21*** |
| Silybin Lipo (100) | 3.07 ± 0.30*** | 11.35 ± 0.50*** | 1.94 ± 0.12***,$^{\$\$}$ | 3.29 ± 0.33***,$^{\$\$}$ |
| Placebo Lipo | 3.57 ± 0.28 | 6.31 ± 0.53 | 0.93 ± 0.17 | 3.60 ± 0.23 |

*Data are expressed as mean ± SEM (n = 6) and analysed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. *p <0.05, **p <0.01 and ***p <0.001 as compared to APAP group. $^{###}$p <0.001 as compared to normal group. $^{\$\$}$p <0.01 as compared to silybin (100 mg/kg) treated. APAP: Acetaminophen; Silybin Lipo (100): Silybin liposomes (equivalent to 100 mg/kg)*

**Table 9. Effect of silybin formulation treatment against APAP-induced alterations in hepatic pro-inflammatory cytokine levels in rats**

| Treatment | Hepatic TNF-α (pg/ mg of protein) | Hepatic IL-1β (pg/ mg of protein) | Hepatic IL-6 (pg/ mg of protein) |
|---|---|---|---|
| Normal | 0.22 ± 0.06 | 0.028 ± 0.006 | 0.15 ± 0.01 |
| APAP Control | 1.41 ± 0.07$^{###}$ | 0.273 ± 0.005$^{###}$ | 0.51 ± 0.03$^{###}$ |
| Silybin (100) | 1.03 ± 0.03** | 0.158 ± 0.015*** | 0.36 ± 0.02*** |
| Silybin Lipo (100) | 0.70 ± 0.07***,$^{$$}$ | 0.090 ± 0.004***,$^{$$}$ | 0.27 ± 0.02*** |
| Placebo Lipo | 0.21 ± 0.06 | 0.030 ± 0.004 | 0.16 ± 0.01 |

*Data are expressed as mean ± SEM (n = 6) and analysed by one-way ANOVA followed by Tukey's multiple range test for each parameter separately. **p < 0.01 and ***p < 0.001 as compared to APAP group. $^{###}$p < 0.001 as compared to normal group. $^{$}$p < 0.05 and $^{$$}$p < 0.01 as compared to silybin (100 mg/kg) treated. APAP: Acetaminophen; Silybin Lipo (100): Silybin liposomes (equivalent to 100 mg/kg); TNF-α: tumor necrosis factor-α; IL: Interleukin*

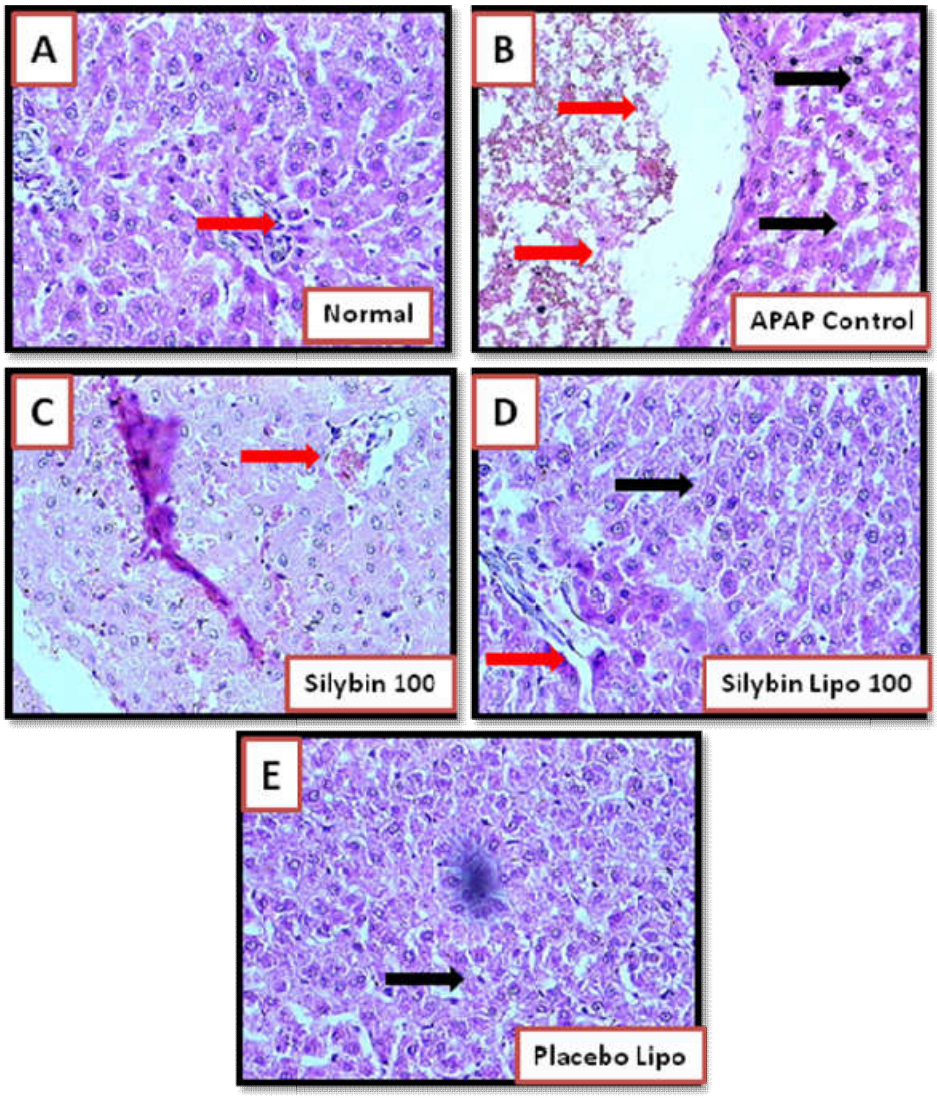


**Fig. 4. Effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat liver**

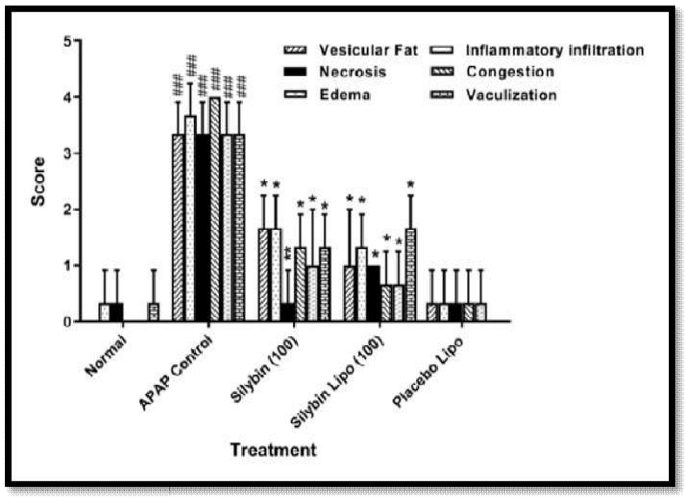


**Fig. 5. Quantitative representation of the effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat liver (J). Data are expressed as mean ± SEM (n = 3) and analyzed by one-way ANOVA followed by the Kruskal-Wallis test for each parameter separately. *p <0.05 and **p <0.01 as compared to APAP group. ###p <0.001 as compared to normal group. Inflammatory infiltration (black arrow), edema (red arrow), and necrosis (yellow arrow). H&E staining at 40 X**

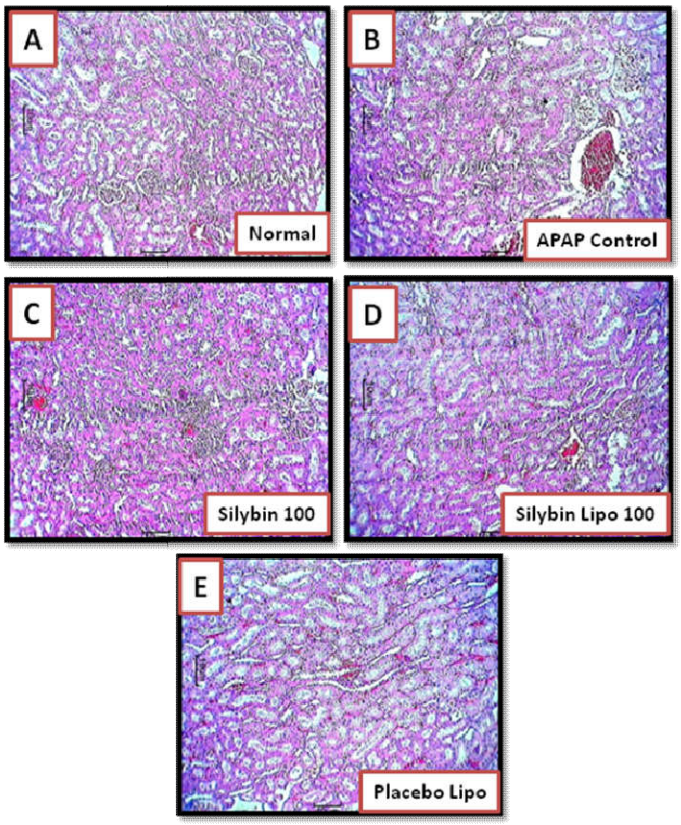


**Fig. 6. Effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat kidney**

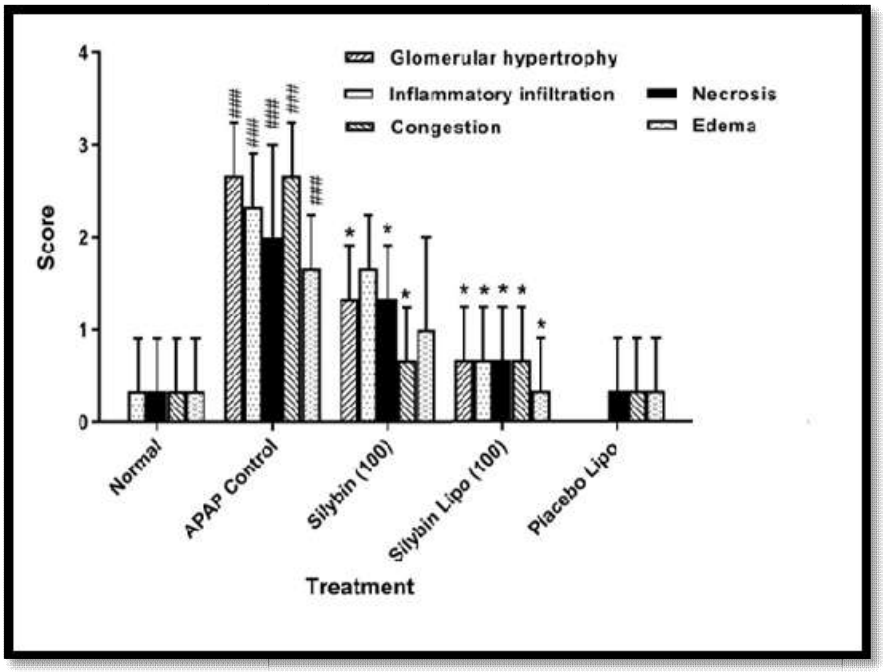


**Fig. 7. Quantitative representation of the effect of silybin formulation treatment against APAP-induced alterations in the pathology of rat kidney (J). Data are expressed as mean ± SEM (n = 3) and analyzed by one-way ANOVA followed by the Kruskal-Wallis test for each parameter separately. *p < 0.05 as compared to APAP group. ###p < 0.001 as compared to normal group. Glomerular hypertrophy (yellow arrow) and inflammatory infiltration (black arrow). H&E staining at 40 X**

capsule, intraluminal cell debris, necrosis, and congestion (Fig. 6). Treatment with silybin (100 mg/kg) & silybin liposomes (eq. to 100 mg/kg) showed a moderate inflammatory cell, few intraluminal cell debris as compared to APAP depicting noticeable inhibition in APAP-induced alterations in renal tissue.

Photomicrograph of sections of kidney of normal (A), APAP control (B), Silybin (100 mg/kg) (C), Silybin liposomes (100 mg/kg) (D), Placebo liposomes (E).

## 4. CONCLUSION

In the present study an attempt was made to optimize the liposomal silybin formulation and target liver for the hepatoprotection. Liposomes were prepared using thin film hydration technique. The parameters optimized were Phosphatidyl choline, Cholesterol and drug concentration. The liposomes were successfully prepared and sustained the drug release for 24hrs. The optimized formulation further evaluated for the hepatoprotective and renal protection action against APAP induced rat liver and renal toxicity models. The silybin liposomes exhibited better *in-vitro* release & *in-vivo* hepatoprotection along with better animal activity & improvement in histopathological changes as compared to silybin.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The experimental protocol was approved by the Institutional Animal Ethics Committee

(registration number 1434/PO/Re/S/11/CPCSEA) and performed in accordance with the guidelines of the Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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